REFERENCE GENE SELECTION FOR REAL-TIME PCR STUDIES OF PERIAPICAL INFLAMMATORY LESIONS

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ABSTRACT

Objective: The appropriate normalization strategy is crucial for accurate real-time PCR data analysis. The aim of this study was to identify the most stable reference genes for expression analyses by real-time PCR in human periapical inflammatory lesions.

Methods: We compared the expression profiles of nine candidate reference genes, B2M, GAPDH, SDHA, YWHAZ, EIF4A2, TOP1, CYC1, ACTB and 18S, using two algorithms, geNorm and NormFinder, on 20 samples categorized as: an experimental group consisting of chronic periapical inflammatory lesions (periapical granulomas (n=6) and radicular cysts (n=7)), and a control group consisting of healthy gingiva tissue (n=7).

Results: According to geNorm algorithm, B2M, SDHA and TOP1 were the three most stable genes and the most optimal combination for normalization in periapical inflammatory lesions. To note, a commonly used reference gene GAPDH was the least stable gene. According to NormFinder algorithm, SDHA, CYC1 and YWHAZ were the most stable genes. 18S was among the least stable genes indicated both by geNorm and Normfinder.

Conclusion: SDHA was the most optimal reference gene, while commonly used GAPDH and 18s showed unstable expression in periapical inflammatory lesions.

Keywords: House-keeping gene, qPCR, periapical granuloma, periapical cyst, SDHA

Introduction

Real-time quantitative PCR (qPCR), known as quantitative polymerase chain reaction, is a widely employed methodology in numerous scientific investigations for the precise and quantitative assessment of gene expression levels. This technique proves to be important in various domains of research, such as molecular biology, genetics and diagnostics, as it offers researchers the ability to scrutinize gene expression patterns, identify indicators of diseases and explore the influence of experimental factors on gene expression. The efficacy of this technique is reliant upon the utilization of reference genes, also referred to as housekeeping genes, which are genes presumed to possess consistent levels of expression across diverse conditions and experimental factors (1).

qPCR is a fast, simple and efficient method for simultaneous measurement of mRNA transcription levels in different types of samples (2). Accurate normalization of data in expression profiles is a crucial step in avoiding variations resulting from differences in quality and amount of starting material, enzymatic efficiencies and transcriptional activities of different types of tissues and cells (3). One of the most widely applied normalization strategies is the usage of reference genes, also known as 'housekeeping genes. An appropriate housekeeping gene shows minimal variability in expression between samples, has great amplification efficiency and is unaffected by the experimental treatment (4, 5). The reference gene serves as the foundation for proper analysis and interpretation, since inappropriate reference gene can result in misleading associations and obscure the statistical significance of alterations in gene expression.

As for now, a small number of studies have been conducted using more than one gene as internal control (6). Moreover, earlier studies have proven that normalization to only one reference gene was unsuitable for a sample of human origin (7, 8). Quantification of several endogenous reference genes simultaneously has been widely accepted as the most reliable method for real time PCR data normalization (9). By employing multiple reference genes, one can discern and accommodate any deviations or irregularities in the levels of expression of each individual reference gene, ultimately resulting in a more dependable standardization of the target gene's expression. This approach aids in mitigating the influence of experimental variables or circumstances that may impinge upon the expression of a solitary reference gene, thereby furnishing a more resilient normalization strategy (1).

The use of multiple reference genes also increases the confidence in the results and reduces the risk of misinterpretation or bias in the analysis. To date, there is no consensus on a single gene/combination of multiple genes that can be used as a reference gene/s for many different types of samples since the expression varies across different tissues, development stages and experimental conditions (1). Thus, prior to use every reference gene is supposed to be validated for particular cell/tissue and experimental conditions.

Human periapical inflammatory lesions, histopathologicallly classified as periapical granulomas and radicular cysts, are lesions of human jaw bone resulting from continuous inflammatory stimuli from infected root canals (10, 11). Accurate differentiation between periapical granuloma and radicular cyst is difficult since it requires surgical removal of lesion tissue. Nowadays, many studies are being carried out on selection of a genetic marker for differentiation between types of human periapical inflammatory lesions which is crucial for proper treatment choice (12). However, neither suitable reference gene nor combination of multiple genes has been selected for performing qPCR analyses in periapical inflammatory lesions.

A number of applets regarding selection of reference genes (9, 13) have been developed recently supporting correct analysis of data in lots of experiments (14-16). Despite obvious differences between possible algorithms to be used for proper reference gene selection, the aim of each algorithm is to present gene expression stability as a numeric value by comparing the expression across a sample set and to rank genes according to their expression stability where the best-ranked ones are taken as the most stable ones (6). The aim of our study was to identify a combination of the most stable reference genes required for correct real-time PCR data normalization in periapical inflammatory lesions. In this study, we assessed a panel of 9 candidate

reference genes on a biologically diverse set of 20 human periapical inflammatory lesions and healthy gingiva samples by employing two algorithms, geNorm and NormFinder. The objective of the study was to compare the expression patterns of 9 common reference genes in order to identify the most stable reference gene for the use in real-time PCR experiments on human periapical inflammatory lesions.

Materials and methods

Samples

This study examined a total of 20 different samples. Six samples from this group were determined to be granulomas, seven samples were determined to be radicular cysts and the remaining seven samples came from healthy gingiva. Immediately after collection, all samples were flash frozen in liquid nitrogen and deposited in a -80°C refrigerator for long-term storage. All procedures performed in study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. As the research was done on human samples, all patients gave their written consent to participate in the study.

RNA isolation was carried out on homogeni-zed samples with *RNeasy Mini Kit* (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The concentration of isolated RNA was quantified by measuring absorbance at 260 nm in ng/µl *(BioSpec nano-UV-VIS Spectrophotometer, Shimadzu Scientific Instruments).* To assess the RNA purity, absorbance at 280 nm and 230 nm was measured and A260/280nm and A260/230nm ratios were calculated. Only high-quality RNA was used for further analysis. cDNA synthesis was performed using *High Capacity cDNA Reverse Transcription Kit* (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Selection of candidate genes and qPCR amplification

Nine candidate genes were chosen from the geNorm system for identification of best reference gene (*Reference gene assays with PerfectProbe by PrimerDesign, UK*) including: beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), eukaryotic translation initiation factor 4A2 (*EIF4A2*), DNA topoisomerase I (*TOP1*), cytochrome c1 (*CYC1*), actin beta (*ACTB*) and 18S ribosomal RNAs (*18S*). Probe-based qPCR was performed for each sample with two replicates using Stratagene Mx3005p qPCR (Agilent Technologies, Santa Clara, USA). The reaction volume was 20 µl and consisted of 10 µl PCR master mix, 1 µl of primer probe mix, 7 µl of RNase/DNase free water and 2 µl cDNA. qPCR amplification was done in following conditions: 95°C for 10 minutes, then 40 cycles of 95°C (15 seconds) and 60°C (1 minute).

Determination of reference gene stability

The Ct (cycle threshold) value in PCR represents the number of cycles required to replicate enough DNA or RNA to be detected (cross a threshold line). Initially, the sample had a higher DNA/RNA content, as indicated by the lower Ct value.

Ct value for each gene was derived by calculating the average value from two replicates. geNorm algorithm-based qbase+ software v. 3.1 and Normfinder algorithm v. 20 were used to determine expression stabilities of candidate reference genes. geNorm algorithm was also used to determine the optimal number of reference genes required for successful normalization.

Statistical analysis

Ct values were tested for normality using Kolmogorv-Smirnov test prior to analysis. The significance level was defined at $P<0.05$. Data was represented as mean and standard deviation. Statistical analysis was performed using SPSS v. 20.2. (Armonk, NY: IBM Corp).

Results

qPCR results

Real-time PCR was used to determine the mRNA levels of nine candidate reference genes across three examined sample groups: periapical granuloma, radicular cyst and healthy gingiva. Derived Ct values were used for determination of gene expression and their stability. Nine candidate reference genes showed a wide expression range (Fig. 1).

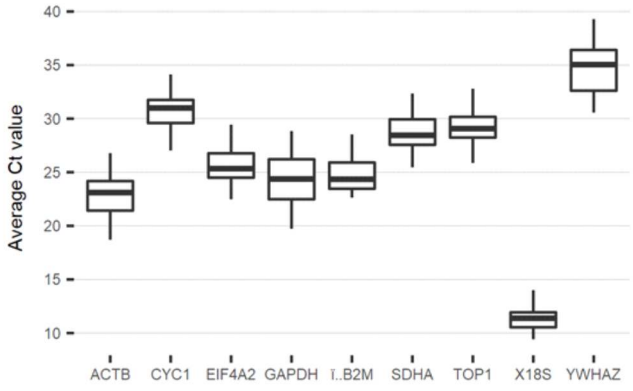

Fig. 1
Expression levels of nine candidate reference genes detected by real-time PCR are depicted on Y-axis as Ct values. Each reaction is done in duplicate. Lower Ct values represent higher expression of the corresponding gene

Ct values ranged from 9.28 to 39.29 and the mean Ct values ranged from 11.57 to 34.67. According to the Kolmogorov-Smirnov test, all genes, except *EIF4A2* and *18S* genes, showed normal distribution in Ct values and they fit both normality criteria (P>0.1, α=0.05). *YWHAZ* was the least expressed gene with Ct values ranging from 30.56 to 39.29. *18S* gene showed highest expression with Ct values ranging from 9.42 to 16.40. Interestingly, *GAPDH* displayed highest variation in mRNA expression compared to the other examined genes, which directly indicated its low expression stability in examined sample groups.

Evaluation of reference gene stability

Two statistical applets (geNorm and NormFinder) were employed in determination of expression stability of nine examined reference genes. In each analysis genes were ranked from most stable to least stable.

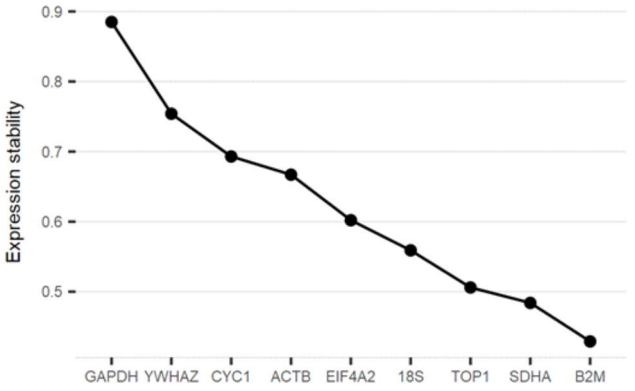

Fig. 2
Average expression stability (M) of nine candidate reference genes by geNorm analysis. The least stable reference gene with highest M value is positioned on the left and the most stable gene with lowest M value is positioned on the right side of the plot. Most stable reference genes were deduced by stepwise exclusion of the least stable genes

geNorm Analysis Nine reference genes were ranked from most stable to least stable based on their stability value (M value), as shown in Fig 2. All tested candidate reference genes demonstrated limited variance in expression, M values being lower that 1.5, which is set as default limit (M<1.5). This indicates a high stability of mRNA expression in experimental conditions. *B2M, SDHA* and *TOP1* were the three most stable genes in examined samples. Surprisingly, *GAPDH* was the least stable gene.

geNorm was also used to determine the optimal number of reference genes required for accurate normalization of qPCR data (Fig 3). Pairwise variations (V) derived by geNorm represent standard

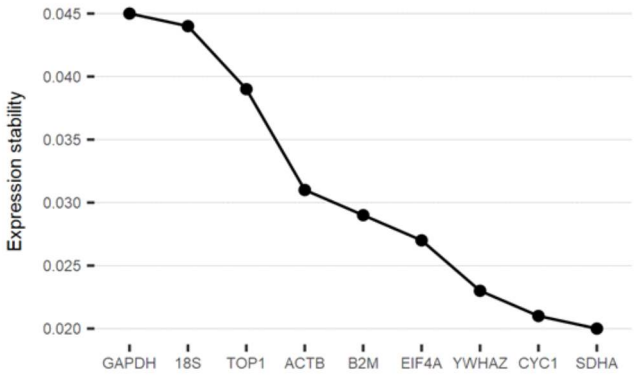

Fig. 3
Pairwise variation analysis of nine candidate reference genes. Optimal number of genes was determined by comparison of Vn/n+1 value (default threshold of variation=0.15)

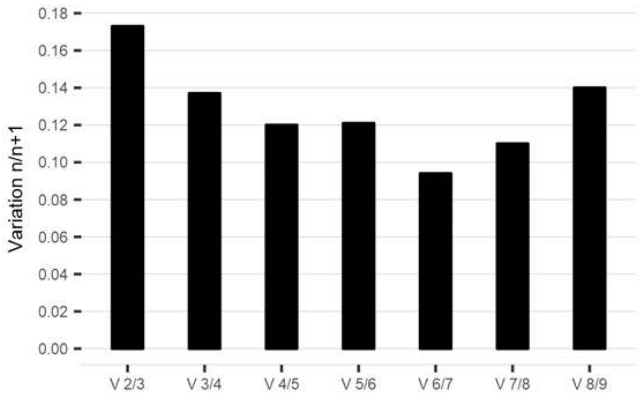

Fig. 4
Expression stability (M) of nine candidate reference genes by NormFinder analysis. The least stable reference gene with highest M value is positioned on the left and the most stable gene with lowest M value is positioned on the right side of the plot.

deviation of log_2-transformed expression ratios of any pair of reference genes. geNorm analysis revealed that the optimal number of genes for normalization in given sample group is three, given that the pairwise variation value V3/4 was lower than threshold value (0.15).

Normfinder Tested reference genes were ranked according to their stability values in given experimental conditions using Normfinder (Fig 4). *SDHA* was listed as the most stable gene with *CYC1* and *YWHAZ* as the second and third most stable genes. Unlike the results generated by geNorm where *YWHAZ* was among two least stable genes, it is found in top three genes in Normfinder. *GAPDH* was ranked as the least stable gene by Normfinder confirming the results generated by geNorm. *18S* was the least stable gene indicated both by geNorm and Normfinder.

Discussion

One of the most appropriate methods for mRNA quantification studies is qPCR analysis)17). However, normalization of expression data requires a suitable gene with stable expression across samples taken from different time points or under different experimental conditions (4). Nowadays, there is a vast number of software and algorithms developed for identification and validation of best reference genes for normalization (18). The choice of combination of reference genes in studies of gene expression in human periapical inflammatory lesions was not previously determined. Moreover, a single reference gene was used for normalization in most gene expression studies in periapical granuloma and radicular cysts without previous determination of the most stable one (19, 20). Even though this problem has been previously addressed, researchers still tend to randomly choose reference genes and, thereby, increase chance of statistical error (8).

By using single gene SD-based approach, scientists assumed that gene expression showed normal distribution across the sample (21). But normality of distribution was rarely assessed during the expression studies. Nowadays, there are algorithms that do not require the reference gene to be normally distributed and the idea of using several reference genes for normalization has been introduced (22). We have previously seen that using only one gene as reference, *SDHA*, gives statistically significant results for studied sample group (periapical granuloma, radicular cyst and healthy gingiva) (23).

Literature review on reference genes used in qPCR analysis in several studies in dental research revealed that the most frequently used housekeeping gene was *β-actin*, which was not among the 3 most stable reference genes in our analysis. Several studies (19, 20, 24, 25) used *β-actin* as the reference of choice in qPCR analyses in odontogenic keratocysts, human gingival fibroblasts, periapical inflammatory lesions (granulomas and cysts), periodontal ligament and similar tissues. Bildt et al. (26) and Kusumi et al. (27) used *G3PDA* as housekeeping gene. Heikinheimo et al. chose *PL2A* as reference gene in their study done on dental follicles and odontogenic keratocysts (28). *GAPDH* was used as a reference gene in the study of radicular cysts and keratocysts done by Hayashi et al. (29).

Different approaches in choosing the best reference genes and to estimate the stability were described previously (18). In our study, we used two free algorithms: geNorm and NormFinder to evaluate expression stability of investigated candidate reference genes. Our study is the first to evaluate the combination of best reference genes for application

in qPCR analysis of periapical granuloma, radicular cysts and healthy gingiva samples. Out of 9 candidate reference genes, following three were among the most stable ones according to geNorm: *B2M*, *SDHA and TOP1. GAPDH* was ranked as the least stable reference gene by geNorm. Even though G*APDH* gave good results in many studies, it is not recommended to be used as reference gene since its expression can vary in different conditions (30, 31).

Unlike geNorm, following genes were found to be the most stable in NormFinder: *SDHA, CYC1* and *YWHAZ. YWHAZ* was considered a poor normalizer in geNorm. This difference can be due to types of algorithms geNorm and NormFinder use, since geNorm excludes poorly expressed genes and NormFinder uses model-gene approach and ranks all tested reference genes independently from their expression level. It is important to mention that according to both, geNorm and NormFinder results, *GAPDH* was among two least stable genes, even though it is among the most used reference gene in previous dental studies in human periapical inflammatory lesions (28).

geNorm algorithm also calculates pairwise-variation (V) value to estimate the adequate number of reference genes to be used. V analysis showed that the V3/4 (0.137) value was just underneath the proposed cut-off value (0.15) indicating that three genes are needed for accurate normalization of expression data (*TOP1, SDHA* and *B2M*).

CONCLUSION

As normalization of qPCR expression data is crucial for analysis of gene expression, proposed genes might help scientists in choosing the best reference gene or combination of multiple genes to use in their studies. Researchers in the field of dentistry must possess knowledge regarding the significance of employing reference genes in order to ensure the reliability of the acquired research findings, as qPCR studies are widely utilized in this particular domain. The study aimed to identify stable reference genes for real-time PCR analysis in human periapical inflammatory lesions by examining the expression profiles of nine candidate reference genes using geNorm and NormFinder algorithms. According to geNorm, B2M, SDHA, and TOP1 were the most stable genes, while GAPDH was the least stable. According to NormFinder, SDHA, CYC1, and YWHAZ were the most stable genes. Thus, SDHA was identified as the most optimal reference gene, while GAPDH and 18S showed unstable expression in periapical inflammatory lesions.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards**.**

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Informed consent Informed consent was obtained from all individual participants included in the study.

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